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The transforming growth factor (TGF) βs are multifunctional polypeptide growth factors which potently inhibit the proliferation of mammary epithelial cells. There is evidence that loss of autocrine regulatory TGFβ signaling promotes mammary carcinogenesis. There is also evidence that tumor cell TGFβs are involved in tumor maintenance and progression by modulating tumor-host interactions that are critical for cancer cell survival, and also by promoting epithelial to mesenchymal transition of epithelial cancer cells. The objective of this proposal is to generate a transgenic breast tumor model in which the mammary epithelial expression of TGFβ1 can be temporally regulated to understand the role of this molecule in progressive sequential stages of mammary neoplasia. In addition, by expressing dominant negative type II TGFβ receptors in MDA-231 human breast cancer cells, we will determine whether blocking autocrine TGFβ in these cells affects their invasive metastatic phenotype *in vitro* and *in vivo*.

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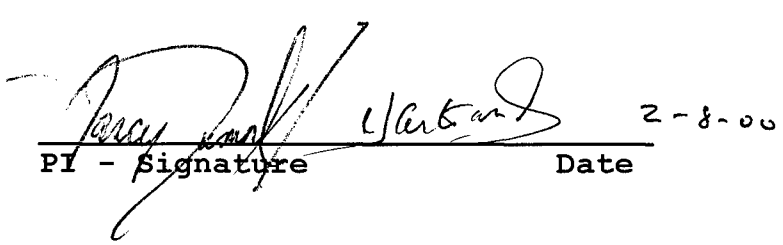

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DAMD17-98-1-8263: 'Temporal control of transforming growth factor (TGF)-β1 on mammary cell multistep transformation'
Nancy Dumont, P.I.
Carlos L. Arteaga, M.D. (Sponsor)

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Introduction

As stated in the original application, the overall goal of this research is to elucidate the complex role of transforming growth factor (TGF) βs in sequential stages of mammary epithelial transformation. This knowledge is of potential importance for the development of rational therapeutic approaches in human breast carcinoma.

Body

Specific Aim 1

The purpose of this Aim was to generate MMTV/tTA + tet-op/TGFβ1$^{S223/225}$ mice. MMTV/tTA founder FVB mice were obtained from Dr. Priscilla Furth (Institute of Human Virology, University of Maryland, Baltimore, MD). During the first year of this award, we generated tet-op/TGFβ1 homozygous founders in a B6D2 mouse strain and cross-bred them with MMTV/tTA FVB mice. To prevent transactivation of the mutant (active) TGFβ1 minigene, pregnant mice were supplemented with doxycycline in the drinking water. Since the submission of the grant, it was reported that this approach was effective in suppressing tTA-mediated transactivation of the tet-op promoter in vivo (Kistner et al. Nature Med. 93:10933, 1996). Results to date can be summarized as follows:

1. The F1 generation of this cross is viable. Mice have been alive for close to a year without any obvious clinical abnormalities.
2. The MMTV/tTA mRNA is expressed in mammary glands, salivary glands, and ovaries. However, the levels of MMTV/tTA mRNA in the mammary gland are very low.
3. The tet-op/TGFβ1 mRNA is detectable in ovarian and salivary gland but NOT in mammary gland tissues.
4. Expression of tet-op/TGFβ1 RNA is suppressible with doxycycline in both salivary gland and ovaries supporting the interaction in vivo of both transgene products.
5. There are no histological abnormalities whatsoever in any of the above mentioned tissues as far as at 8 months of age.

Revised approach. We hypothesize that there are two potential reasons to explain our inability to detect the tet-op/TGFβ1 transgene is the mammary gland of the F1 mice: [a] the low level of expression of the MMTV in this founder line; and [b] the possibility that upon removal of doxycycline from the mouse water, those mammary epithelial cells in which the mutant active TGFβ1 is expressed, are eliminated by apoptosis. To test or account for these possibilities, we have revised our approach as follows:

1. New MMTV/tTA founder mice are being generated at the Vanderbilt-Ingram Cancer Center Transgenic Mouse and ES Cell Core Facility. In addition, we have requested other MMTV/tTA founder lines with more robust MMTV expression in the mammary gland. These will be provided by Dr. Lewis Chodosh (University of Pennsylvania, Philadelphia, PA).

2. We are establishing mouse mammary epithelial cell lines from MMTV/tTA + tet-op/TGFβ1 mice. These are being generated in the constant presence of 1 μM tetracycline (tet) to maintain the tTA suppressed. Once these lines are established, we will proceed to remove tet and examine for tet-op/TGFβ1 expression by RT-PCR and other immunological methods. This will confirm that there is expression of the second transgene product but that the temporal window in which this occurred in the mice was missed by the in vivo studies.

Specific Aim 2

This Aim proposed to study the effect of mammary TGFβ1 overexpression on different stages of breast transformation in MMTV/neu + TGFα bigenic mice. These studies are on hold until an appropiate MMTV/tTA + tet-op/TGFβ1 bigenic mouse is generated.

Revised approach. However, due to the enormous complexity of generating a mouse between these two bigenics bearing FOUR different transgenes, two alternative equally informative and faster approaches are being considered:

1. Crossing the MMTV/tTA + tet-op/TGFβ1 bigenic mice with MMTV/mutant neu mice (described in Siegel et al. EMBO J. 18:2149-2164, 1999). In these mice, the mutant neu transgene product exhibits a deletion of a short cysteine-rich yuxtamembrane region in neu, resulting in constitutive phosphorylation/activation of the neu tyrosine kinase. Mice develop stochastic mammary tumors with a T50 of <140 days. Homozygous FVB founder MMTV/mutant neu mice have already been provided by Dr. William Muller (McMaster University, Ontario, Canada)

2. The second alternative will be to treat the bigenic MMTV/tTA + tet-op/TGFβ1 mice with the carcinogen 7,12-dimethylbenzanthracene (DMBA) given by orogastric tube at 1 μg weekly x4. In FVB mice, treatment with these doses and intervals of DMBA results in 100% mammary tumor formation by 20 weeks, with the resulting breast tumors going through the same histopathological changes described for human breast cancer (Medina D. J. Mammary Gland Biol. Neopl. 1:5, 1996; Li et al. Mol. Carcinogenesis 14:75-83, 1995).

Specific Aim 3

This Aim proposed to test the effect of antisense TGFβ1 and antisense TGFβ2 on MDA-231 human breast cancer cells. As indicated in the original 'statement of work', the antisense vectors were generated and transfected into MDA-231 cells. However, we have been unable to generate stable transfectants with sustained expression of the antisense. We suspect that this is toxic to the cells as suggested by a recent report (Rauh-Adlemann et al. Proc. Amer. Assoc. Cancer Res. 39:971a, 1998).

Revised approach. Recent reports, however, suggest that blocking the type II TGFβ receptor (TβRII) in tumor cells might be a more effective way of disrupting

autocrine TGFβ in tumor cells (Oft et al. Curr. Biol. 8:1243-1252, 1998). Therefore, as an alternative we are pursuing transfection of expression vectors for dominant negative TGFβ type II receptor (TβRII) into MDA-231 cells. GFP containing vectors encoding a truncated TβRII (lacking the cytoplasmic domain) or a Lys-to-Arg ATP site-mutant TβRII have been obtained from Dr. Martin Oft (University of California, San Francisco). Stably transfected pools have been generated and are being characterized now by examining the activity of TGFβ-induced luciferase reporter constructs after treatment or not with exogenous TGFβ1.

This approach is more robust than the originally proposed antisense strategy in that it will avoid compensatory increases of TGFβ isoforms other than that targeted by the antisense vector. By eliminating autocrine TGFβ signaling, the dominant negative receptor approach will block autocrine function of all three TGFβ isoforms. Cells transfected with mutant TβRII, will be subjected to the same experimental endpoints proposed in the original Aim 3.

Reportable outcomes

Dumont N. Genetic and epigenetic contributions to colorectal cancer. *APMIS* 107:711-722, 1999

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Genetic and epigenetic contributions to colorectal cancer

Review article

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Dumont N. Genetic and epigenetic contributions to colorectal cancer. APMIS 1999;107:711–22.

Both genetic and epigenetic factors contribute to the development of colorectal cancer. Specific genetic changes in proto-oncogenes, tumor suppressor genes, and DNA mismatch repair genes have led to a genetic model of colorectal tumorigenesis. Recent data highlight the importance of the TGF-β signaling pathway in regulating the progression of colorectal cancer. The loss of the tumor suppressor activity of this pathway as well as the potentially cooperative genetic aberrations involving *APC*, *K-ras*, and *p53* are reviewed in the context of the multi-step adenoma-carcinoma sequence that characterizes the development of colorectal tumorigenesis. In addition, contributing epigenetic factors including age, diet, angiogenesis, and immune response are also discussed. Combining our knowledge of the genetic and epigenetic events implicated in this disease may allow a broader understanding of the pathogenesis of colorectal cancer and hence the design of better anti-tumor interventions.

Key words: Colorectal carcinoma; oncogene; tumor suppressor gene; mismatch repair; transforming growth factor-β; epigenetic.

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Like many cancers, colorectal cancer arises by acquisition of genetic alterations that result in cellular transformation. Based on these alterations, a genetic model for colorectal tumorigenesis has been proposed (1). However, in addition to genetic changes, several epigenetic factors have also been shown to contribute to the development of colorectal cancer. This paper reviews genetic as well as epigenetic contributions to colorectal cancer, both of which present opportunities as potential therapeutic targets.

GENETIC ABERRATIONS AND RELATED HISTOPATHOLOGICAL FEATURES

The development of colorectal cancer is a multistep process involving a series of genetic changes in the colonic mucosa that lead sequentially to hyperplasia, adenoma, carcinoma, and metastasis. Numerous genes, including proto-oncogenes, tumor suppressor genes, and DNA mismatch repair genes, have been implicated in the genesis of colon cancer. The discrete genetic changes currently perceived as fundamental to the multistep process of colorectal tumorigenesis are illustrated in Fig. 1. The tumorigenic process is initiated when a cell of the normal epithelium presumably undergoes a genetic change that conveys a selective growth advantage. This predisposes it to additional mutations, each of which confers further malignant potential, thereby leading to the clonal expansion of this cell (1). Thus, neoplasms of the colon are clonal in nature in that they arise from a single cell. Although sporadic mutations account for the majority of colorectal cancers, there are two hereditary syndromes in which a strong tendency to develop colorectal cancer is transmitted by dominant inheritance: Familial Adenomatous Polyposis (FAP) and Hereditary

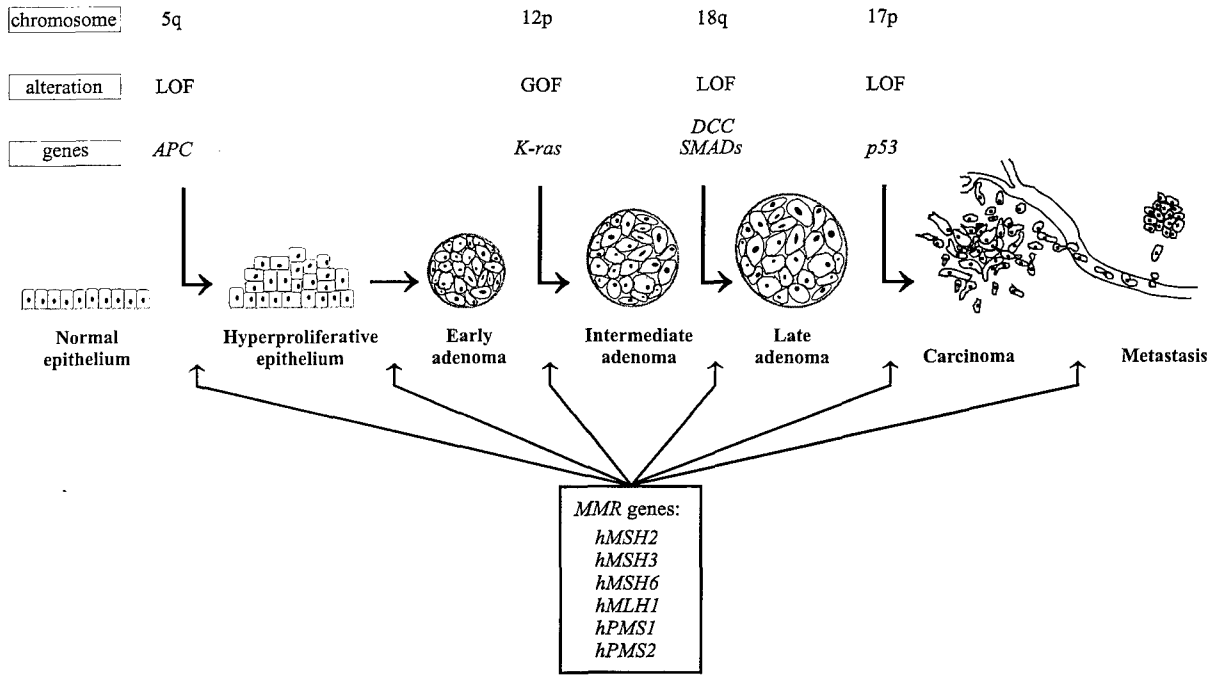


Fig. 1. Genetic changes associated with colorectal tumorigenesis. This process is accelerated by MMR deficiency (see text for details). Abbreviations: LOF, loss of function; GOF, gain of function; MMR, mismatch repair. Reproduced from Kinzler & Vogelstein (2) with modifications.

Nonpolyposis Colorectal Cancer (HNPCC) (2). FAP is a syndrome in which an inherited defect in the *adenomatous polyposis coli* (*APC*) gene leads to the development of multiple benign polyps throughout the colon, some of which slowly progress to invasive lesions. Thus, defects in *APC* initiate the tumorigenic process, but additional mutations, such as those illustrated in Fig. 1, are required for tumor progression. In contrast to FAP, HNPCC is a syndrome characterized by the rapid progression of colorectal tumors due to inherited defects in DNA mismatch repair (*MMR*) genes. Although the tumors from patients with HNPCC go through a series of mutations similar to those described in Fig. 1, additional mutations, unique to HNPCC, have been described. Evidence for the role of the genes most commonly implicated in sporadic and hereditary colorectal tumors are discussed individually below.

APC

One of the earliest steps in the development of colorectal cancer is loss of function of the tumor suppressor gene, *APC*. This gene was first identified as the gene responsible for FAP by demonstrating cosegregation of mutant *APC* alleles in affected kindreds (3, 4). However, the chromosome 5q region containing this gene is also frequently affected by loss of heterozygosity (LOH) events in colorectal adenomas and carcinomas from patients without polyposis (5–7). In fact, over 70% of sporadic colorectal cancers are believed to involve mutations in *APC* (8). Studies have shown that somatic mutations identified in sporadic tumors are similar to those observed in the germline of patients with FAP, often involving codon 1309 located in the mutation cluster region of the gene (9). These mutations have been identified in adenomas as small as 5 mm, consistent with the idea that mutations in the *APC* gene may be the initiating genetic event in many tumors (7). This is supported by the fact that excision of adenomatous polyps significantly reduces the incidence of cancer development (10). Further support for the role of *APC* in the development of polyps and colorectal cancer stems from studies of a mouse genetic model for FAP known as *Min* (for *m*ultiple *i*ntestinal *n*eoplasia). The *Min* mutation, like those in many FAP patients, causes

premature truncation of the APC protein, and mice heterozygous for the *Min* allele develop multiple adenomatous polyps and cancers in their intestine (11).

With respect to histopathological features, *APC* mutations lead to dysplastic lesions affecting the tube-shaped epithelial foldings of the colon referred to as crypts. Foci of dysplastic aberrant crypts are the earliest identifiable lesions in colorectal tumorigenesis, and are believed to be the precursors of adenomas (12). In FAP patients, different types of *APC* mutations are associated with different clinical features despite the fact that virtually all mutations result in C-terminally truncated APC proteins (2). These differences are manifested primarily in extracolonic sites, but may also be manifested by an attenuated form of FAP in which patients develop fewer polyps (13). This phenotype is also observed in the *Min* mouse where, depending on the inbred strain carrying the *Min* allele, wide variations in polyp number are seen. Linkage analysis has demonstrated that much of the variation is due to a single locus, named *MOM-1* (for *m*odifier of *M*in) (14), which encodes a secreted phospholipase A2 (15).

Although the exact mechanism by which *APC* mutations cause abnormal growth of colorectal epithelial cells is not clear, the fact that the majority of somatic and germline mutations in *APC* generate truncated APC proteins that lack a β-catenin-binding domain suggests that the interaction between APC and β-catenin may be important (16). Indeed, studies have shown that APC and glycogen synthase kinase 3 regulate cytoplasmic β-catenin levels by promoting its degradation (17). Inactivation of *APC* in colorectal cells allows β-catenin to accumulate and complex with T-cell factor 4, leading to activation of transcription and deregulated cell growth (18). The importance of APC and β-catenin in the development of colorectal cancer is further illustrated by the finding that β-catenin is mutated in a subset of colorectal cancers that lack somatic mutations in *APC* (19, 20). In addition to its role in regulating β-catenin, APC may also be involved in regulating apoptosis. Studies have shown that expression of wild-type APC in colorectal epithelial cells with *APC* mutations results in cell death (21). Thus, this may be another mechanism by which inactivation of *APC* leads to deregulated cell growth.

K-ras

Although the three *ras* genes, K-*ras*, H-*ras*, and N-*ras*, are highly homologous, and believed to be expressed at relatively equivalent levels in the colonic mucosa, only K-*ras* plays a significant role in the development of colorectal cancer. Mutations in K-*ras* can be identified in 50% of colorectal cancers (22), and occur most frequently in codon 12, with fewer mutations found at codons 13 and 61 (23). Because K-*ras* is an oncogene, mutation of one allele is enough to produce an effect. These mutations affect the ability of p21ras to interact with the ras GTPase-activating protein, causing p21ras to remain in the active GTP-bound state. As a result, the growth and differentiation signal transduction pathways that include p21ras are constitutively activated, leading to a continually growth-stimulated state.

The frequency of *ras* mutations appears to be correlated with two histopathological features: increased tumor size and dysplasia. Studies have shown that 50% of adenomas greater than 1 cm in diameter harbor K-*ras* mutations compared to only 10% of adenomas less than 1 cm (22). When adenomas are distinguished from one another with respect to the degree of dysplasia, *ras* mutations are more prevalent in tumors with increased dysplasia (24). The higher prevalence of *ras* mutations in later stage adenomas and carcinomas suggests that these mutations may arise in one cell of a small preexisting adenoma causing it to progress to a larger and more dysplastic adenoma, with greater risk of subsequent progression to cancer. This is consistent with the fact that hyperplastic cells containing mutant *ras* genes, unlike their dysplastic counterparts with mutant *APC* genes, have little or no potential to form clinically important tumors and may eventually regress through apoptosis (25).

DCC, Smad4, and the tumor suppressor activity of the TGF-β pathway

LOH affecting the long arm of chromosome 18 can be detected in more than 70% of primary colorectal cancers, in about 50% of advanced adenomas, and infrequently in earlier stage adenomas, suggesting that loss in this region is a relatively late event (22). Such losses are also correlated with greater mortality and increased propensity for metastatic spread (26, 27). Ef-

forts to identify a candidate tumor suppressor gene from 18q led to the discovery of a gene termed *DCC* (for *d*eleted in *c*olorectal *c*ancer) (28). The *DCC* gene encodes a transmembrane protein of the immunoglobulin superfamily. The predicted structural similarity of DCC to the N-CAM family of cell-surface adhesion molecules suggested that it might function in differentiation pathways and cell fate determination through cell-cell and/or cell-extracellular matrix interactions (29). Therefore, it was hypothesized that loss of cell-cell contact might explain the enhanced metastasis observed in patients with loss of *DCC* (29). However, more recent studies have shown that inactivation of the murine *DCC* gene does not affect the proliferation or differentiation of intestinal epithelial cells, nor does it affect the morphogenesis of colonic crypts and villi (30). Moreover, introduction of the null *DCC* allele into the germ line of the *Min* mouse does not accelerate the progression of, or modify the phenotype of polyps initiated in the *Min* mice (30). Instead, the phenotype of mice lacking a functional *DCC* gene resembles that of netrin-1-deficient mice, with defects in axonal projections and brain development (30). These findings fail to support a tumor suppressor function for *DCC* in the development of colorectal cancer, and are inconsistent with studies in which reduction or loss of *DCC* RNA has been observed in cell lines or xenografts derived from human colon carcinomas (28, 31).

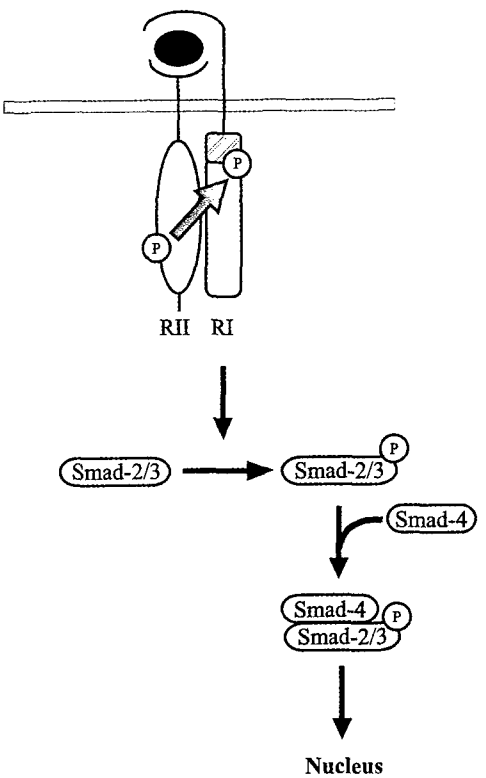
The discrepancy between these results may be due to differences in the pathogenesis of colorectal cancer between mice and humans. Alternatively, LOH of 18q21 may not only affect the *DCC* gene, but neighboring genes as well, one or more of which may be the target of inactivation during colon tumor progression (30). Indeed, other candidate tumor suppressor genes, including *Smad2* and *Smad4*, have been identified on chromosome 18q21 (32, 33). Both *Smad2* and *Smad4* belong to the *SMAD* gene family involved in the signal transduction pathways activated through the TGF-β family of receptors (34). The TGF-βs are important regulators of cell growth and differentiation (35). Escape from the growth regulatory effects of TGF-βs is common among many different cancers (35, 36), including colorectal cancer (37). Recent studies have shown that inactivation of the *Smad4* gene in $APC^{\Delta 716}$ knockout mice, which have a phenotype similar to that of the *Min* mice, results in malignant progression of the intestinal tumors at a much earlier stage than that observed in the simple $APC^{\Delta 716}$ knockout mice (38). Mice heterozygous only for the *Smad4* knockout show no apparent tumor phenotype, indicating that the *Smad4* gene is a suppressor of tumor progression, but not of tumor initiation (38). The fact that inactivation of *Smad4* in $APC^{\Delta 716}$ knockout mice enhances tumor progression, while inactivation of *DCC* in a similar mouse model does not, suggests that the tumor suppressor gene associated with LOH on 18q21 is more likely to be *Smad4*. In support of this, mutations in the *Smad4* gene have been identified in human colorectal cancers in vivo (33, 39, 40), and in familial juvenile polyposis, which, like FAP, is a syndrome characterized by a predisposition to hemartomatous polyps and gastrointestinal cancer (41).

A role for *Smad4* in the malignant progression of colorectal tumors is consistent with previous findings that have implicated other members of the TGF-β pathway in colorectal tumorigenesis (summarized in Fig. 2). For example, as previously mentioned, *Smad2* is a candidate tumor suppressor gene also located on 18q21. Inactivating missense mutations and deletions of the *Smad2* gene have been detected in sporadic colorectal carcinomas (32, 42). In addition to Smad2 and Smad4, Smad3 is another member of the SMAD family of proteins involved in mediating TGF-β signaling. Although mutations in the *Smad3* gene have not yet been detected in human colorectal cancer (43, 44), LOH of *Smad3* has been detected in 2 (1 sporadic and 1 HNPCC) of 17 colorectal cancers examined (44), and a recent study has reported that inactivation of the *Smad3* gene in mice leads to the development of metastatic colorectal cancer (45). Upstream of the SMAD signaling proteins, mutations in the TGF-β type II receptor (TGF-βRII) have been associated with microsatellite instability in both colon cancers and colorectal carcinoma cell lines (46–48). Restoration of TGF-βRII expression by gene transfection has been found to reverse the transformed phenotype (49). There is also evidence that transfection of TGF-β-responsive human colon carcinoma cells with a TGF-β1 antisense expression vector increases their tumorigenicity (50). These data taken together highlight the im-

portance of the TGF-β signaling pathway in regulating the progression of colorectal cancer.

p53

The chromosomal region most frequently affected by LOH in colorectal cancers is 17p (51), a region which includes the tumor suppressor gene, *p53*. Sequence analysis of the remaining *p53* allele from a large number of colorectal carcinomas in which 17p was affected by LOH revealed that missense mutations were present in over 80% of the cases (52, 53), indicating that loss of *p53* function is an important step in the development of colorectal cancer. Although *p53* mutations are extremely prevalent in many advanced colorectal cancers, they are much less frequent in earlier stages of carcinogenesis, and rare in both adenomas and polyps (22). Moreover, patients with germline mutations in *p53* do not develop polyposis, nor are they at risk for developing colorectal cancer (54). This suggests that, although *p53* plays an important role in colorectal carcinogenesis, unlike *APC* it cannot initiate the process. Likewise, *p53*-deficient mice are prone to develop cancers, but an initiating event is required for tumor development (55, 56). Moreover, the incidence of colorectal cancer in these animals is low, indicating that loss of *p53* function alone does not cause transformation (57). This is consistent with the role of p53 as a cell-cycle checkpoint regulator (58). Abrogation of either p53-



TGF-β signaling component	Role in colorectal tumorigenesis	Ref.
TGF-β	-Loss of sensitivity of colon cancer cells to growth inhibitory effects of TGF-β	37
	-Antisense TGF-β1 increases tumorigenicity of human colon cancer cells	50
TGF-βRII	-Mutated in colon cancers and in colorectal carcinoma cell lines with MSI	46-48
	-Restoration of RII expression reverses transformed phenotype	49
Smad2	-Mutated in sporadic colorectal cancer	32,42
Smad3	-*Smad3-/-* mice develop metastatic colorectal cancer	45
	-LOH in 2 (1 sporadic and 1 HNPCC) of 17 cancers examined	44
Smad4	-Inactivation of *Smad4* in $APC^{\Delta 716}$ mice enhances tumor progression	38
	-Mutated in human colorectal cancers *in vivo*	39-40
	-Mutated in familial juvenile polyposis	41

Fig. 2. The TGF-β signaling pathway and its role in colorectal tumorigenesis. TGF-β (represented as a circle) elicits its effects through binding to specific cell-surface receptors denoted type I (RI) and type II (RII) TGF-β receptors, both of which are transmembrane serine/threonine kinases. TGF-β binds directly to RII, which is a constitutively active kinase. The ligand-bound RII then recognizes RI, leading to the formation of a heteromeric complex, allowing RII to phosphorylate and thereby activate RI. The activated RI phosphorylates Smad-2 or Smad-3, which then associates with Smad-4. This complex translocates to the nucleus where it can initiate gene transcription. TGF-β, RII, Smad-2, Smad-3, and Smad-4 have all been implicated as possible tumor suppressors in colorectal cancer, as summarized in the table above (see text for details). Abbreviations: MSI, microsatellite instability; LOH, loss of heterozygosity. Signaling pathway reproduced with modifictions from Massague (34).

dependent cell-cycle arrest or apoptosis could allow cells that have incurred mutations and are not fit to progress through the cell cycle to do so anyway, resulting in tumor progression.

With respect to clinical features, the presence of mutant p53, as determined by increased immunohistochemical staining or by analysis of gene sequence, is correlated with poor survival, as well as increased cancer recurrence rates (59, 60).

MMR genes

DNA mismatch repair plays a prominent role in the correction of replicative mismatches which escape DNA polymerase proofreading. Three genes, *MutS*, *MutL*, and *MutH,* are central to the correction of replication errors in E. coli (61). In humans, germline mutations in the *MutS* homologue, *hMSH2* (62, 63), or in any of the three MutL homologues, *hMLH1*, *hPMS1*, and *hPMS2* (64–66), have been identified as being responsible for HNPCC. These reports suggest that mutations in *hMSH2* and *hMLH1* together account for the majority of HNPCC, while mutations in *hPMS1* and *hPMS2* are less frequently observed. A characteristic feature of tumors arising in individuals with HNPCC is the presence of microsatellite instability. Microsatellites are regions consisting of single, dinucleotide, or trinucleotide repeats that are widely distributed throughout the genome. These sequences are prone to replication errors due to their repetitive structure, which favors slippage during replication. Thus, when *MMR* genes are mutated, errors arising during DNA replication are less efficiently corrected, resulting in a replication error-prone (RER+) phenotype. Affected cells accumulate errors (mutations) at a much greater rate than normal cells (67). This is manifested clinically by a much faster progression of the disease. In contrast to sporadic colorectal cancers, which may take 10 to 15 years to develop, patients with HNPCC have been found to develop cancers within 2 years after a normal colonoscopy (68).

Microsatellite instability is not only a characteristic feature of HNPCC tumors, it is also observed in a subset (about 17%) of sporadic colorectal cancers (69–73). Sporadic tumors with microsatellite instability share common clinical and histopathological features with HNPCC tumors. They are usually located in the proximal colon (71–74), are associated with extracellular mucin production (73), poor differentiation (71–73), and diploidy (69, 72). In addition, they are less likely to have LOH at known tumor suppressor gene loci on chromosomes 5q, 18q, and 17p (74). On the other hand, inactivation of TGF-βRII due to frameshift mutations within coding microsatellite sequences occurs frequently (47, 48). Although defects in the same *MMR* genes that are affected in HNPCC have been identified in sporadic colorectal cancers with microsatellite instability (75), substantial differences in the nature and incidence of these mutations have been reported, suggesting that the molecular mechanisms underlying instability in the sporadic cases differ from those in HNPCC (2, 76). However, mutations in *hMSH3* and *hMSH6*, two other *MutS* genes initially identified in sporadic colorectal tumors with microsatellite instability (77, 78), have recently been reported in HNPCC patients. This suggests that the molecular mechanisms underlying instability may be similar in tumors with microsatellite instability regardless of their sporadic or hereditary nature (79).

EPIGENETIC FACTORS THAT CONTRIBUTE TO COLORECTAL CANCER

Age and diet. Consistent with the multi-hit hypothesis for the development of cancer, the incidence of colorectal cancer increases with age, as greater numbers of mutations are accumulated with time (80, 81). Although genetic aberrations and hereditary disorders play a critical role in the development of colorectal cancers (section above), the fact that the incidence varies tremendously according to geography suggests that other factors must also be involved (82). One of the major differences in lifestyle between cultures (which may explain geographic differences in colorectal cancer incidence) is diet (83). A number of dietary components have been implicated as possible factors in the development of colorectal cancer. The major factors include overall caloric intake, fat content of the diet, and fiber intake. The number of calories consumed per day and the fat content in the diet have been consistently positively correlated with the

risk of developing colorectal cancer (80). The mechanism by which a high-fat diet enhances tumor formation may be related to high fecal bile acid levels which stimulate mucosal epithelial proliferation. In contrast, vegetable and fiber consumption seem to have a protective effect (84, 85). Fiber may work either by increasing the stool transit time and thereby decreasing contact with fecal contents, by binding luminal toxic compounds, or by providing fuel for colonic bacteria that produce short-chain fatty acids that may inhibit proliferation and promote apoptosis (86). Consistent with the protective effects of dietary fiber, the intake of selenium, an essential trace mineral found in cereal grains, has also been found to be inversely correlated with the incidence of colorectal cancer (87–89). Since selenium is a cofactor for glutathione peroxidase, which participates in preventing free radical damage to tissues, part of selenium's protective effect may be due to a reduction in free radical damage.

Angiogenesis. In addition to the risk factors associated with lifestyle, physiological processes such as angiogenesis also contribute to the pathogenesis of colorectal cancer. Studies have shown that in order for a tumor to grow beyond a few millimeters in diameter, the formation of new blood vessels is required to provide nutrients and a means of eliminating metabolic waste products (90). As tumors get larger, the center of the tumor often becomes hypoxic due to inadequate vascularization, leading to cell death within the hypoxic center. Because hypoxia can induce apoptosis in a p53-dependent manner (91), low oxygen conditions can provide a selective advantage for cells carrying mutations in p53, allowing escape from apoptosis. This may be particularly important in colorectal cancer because p53 mutations are prevalent, and occur late in the adenoma-carcinoma sequence. Thus, by escaping apoptosis, tumor cells bearing p53 mutations within a colorectal carcinoma retain their proliferative capacity, thereby promoting tumor expansion. In addition, since p53 expression results in the secretion of inhibitors of angiogenesis (92, 93), selection of p53 mutations by hypoxic conditions could lead to loss of expression of antiangiogenic factors, allowing growth of new blood vessels, thereby favoring further expansion of the tumor.

Angiogenesis is not only essential for the expansion of the primary tumor, it is also required for the establishment and growth of metastases at distant sites. In fact, there is evidence that systemic suppression of angiogenesis can maintain micrometastases dormant as a result of a balance between proliferation and apoptosis (94). Therefore, the degree of angiogenesis may be an important factor in determining tumor behavior and the propensity to metastasize. Indeed, in patients with colorectal cancer, angiogenesis has been correlated with a higher recurrence rate and diminished survival (95).

Immune response. Another epigenetic factor important in the development of colorectal cancer is ineffective immune response. Unlike virally or chemically induced tumors, spontaneous tumors, such as those arising in the colon, elicit a weak immune response. Although mutant proteins encoded by oncogenes (K-*ras*) or tumor suppressor genes (*APC*, *DCC*, *Smad4*, *Smad2*, *tgf-βRII*, *tgf-β1*, *p53*) that have undergone mutations can be recognized as tumor-specific antigens, their recognition delivers only one of the two signals required for T-cell-mediated immunity. This is due to the fact that very few professional antigen-presenting cells (dendritic cells, macrophages, and B-cells) are present in colorectal tumors (96). Thus, most of the mutant proteins are processed and presented as antigenic peptides bound to class I MHC on the surface of colon cancer cells. Since these cells are not professional antigen-presenting cells, they lack the costimulatory signal (B7) that must be recognized by T-cells in order to elicit an immune response. Recognition of foreign antigen in the absence of a costimulatory signal leads to T-cell anergy. Consequently, although a large number of T-lymphocytes have been identified in primary colorectal tumors (97), T-cell-mediated immunity is ineffective in eliminating tumor cells. In contrast, natural killer cells have a spontaneous cytotoxic capacity against tumor cells. However, these cells are either not found or are only present in low numbers in colorectal cancers (96, 98). In addition, studies have shown that MHC expression on professional antigen-presenting cells within colorectal tumors is often lost, thereby further compromising immunity against tumors (99).

Related to immune function, epidemio-

logical studies have shown that chronic use of aspirin results in a reduced risk of colorectal cancer (100), and that treatment of FAP patients with non-steroidal anti-inflammatory drugs (NSAIDs) results in regression of rectal polyps (101). These studies suggest that inflammation may contribute to the development of colorectal cancer. NSAIDs mediate their effects by inhibiting two enzymes, COX-1 and COX-2, which are responsible for eicosanoid synthesis. Analysis of COX-2 mRNA in colon cancers and adenomatous polyps revealed increases of 86% and 43%, respectively, compared to in normal mucosa from the same patients (102). Consistent with elevated COX-2 levels, prostaglandin E2 levels have also been found to be elevated in colon cancers and polyps (103, 104). Further support for the role of COX-2 in the development of colorectal cancer has been obtained using an *APC* knockout mouse with a phenotype similar to that of the *Min* mouse. Inactivation of the *COX-2* gene in these mice resulted in an 86% decrease in intestinal and colonic polyps compared to control animals (105). In addition, when animals heterozygous for *APC*, but having normal *COX-2*, were treated with a novel specific inhibitor of COX-2 enzyme activity, polyp numbers decreased by 50–60% compared to a 26% decrease when they were treated with the NSAID sulindac (105). Moreover, feeding a selective COX-2 inhibitor to rats has been reported to completely prevent the development of chemically induced colon tumors in 93% of animals (106). These data suggest a direct role of COX-2 in the development of colorectal cancer.

There is evidence that the mechanism by which COX-2 overexpression contributes to colorectal cancer involves a decrease in apoptosis due to induction of Bcl-2 by prostaglandin E2, rather than an increase in inflammation, as initially suggested (107, 108). In addition, COX-2-overexpressing colon cancer cells have been shown to produce high levels of angiogenic factors, which stimulate both endothelial migration and tube formation (109). Thus, COX-2 may also act as a tumor promoter via stimulation of angiogenesis. This is consistent with a previous study which has shown that the antitumor effect of the COX inhibitor diclofenac was due to an antiangiogenic effect (110).

CONCLUSION

Since many genetic aberrations contribute to the development of colorectal cancer, each affected gene could potentially be a good therapeutic target. Identification of these genetic aberrations has provided an opportunity to use different strategies to deliver normal copies of defective tumor suppressor genes to affected tissues, or to inactivate oncogenes. Although these avenues hold promise, not all of the genetic aberrations described above occur in all individuals with colorectal cancer. In contrast, epigenetic factors do indeed contribute to the development of all colorectal tumors and thereby provide additional opportunities for intervention. Therapies targeting both genetic and epigenetic aberrations could be combined to provide a broader and more effective therapy for patients.

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Tumor Promoting Effects of the Transforming Growth Factor (TGF)-βs

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List of Abbreviations:

TGF-β: transforming growth factor-β; EMT: epithelial mesenchymal transition; TβRII: TGF-β receptor type II; MSI: microsatellite instability; PTHrP: parathyroid hormone-related protein; PAI-1: plasminogen activator inhibitor; uPA: urokinase plasminogen activator; TβRI: TGF-β receptor type I; JNK: c-Jun N-terminal kinase; ILK: integrin-linked kinase; MMP: matrix metalloproteases; VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor; PI-3K: phosphatidylinositol-3 kinase

Abstract

The transforming growth factor (TGF)-βs are potent growth inhibitors of normal epithelial cells. In established tumor cell systems, however, the preponderant experimental evidence suggests that TGF-βs can foster tumor-host interactions which indirectly support the viability and/or progression of cancer cells. The timing of this 'TGF-β switch' during the progressive transformation of epithelial cells is not clear. More recent evidence also suggests that autocrine TGF-β signaling is operative in some tumor cells and can also contribute to tumor invasiveness and metastases independent of an effect on non tumor cells. The dissociation of antiproliferative and matrix-associated effects of autocrine TGF-β signaling at a trascriptional level provides for a mechanism(s) by which cancer cells can selectively utilize this signaling pathway for tumor progression. Data in support of the cellular and molecular mechanisms by which TGF-β signaling can accelerate the natural history of tumors will be reviewed in this section.

Introduction

Although the TGF-βs can be tumor suppressive [1], there is increasing evidence that TGF-β secretion by tumor cells and/or stromal cells within the peritumoral microenvironment can contribute to tumor maintenance and progression. How then, can TGF-βs be both tumor suppressive and tumor promoting? This apparent paradox is reconciled by a study which showed that, in a mouse skin model of chemical carcinogenesis, targeted expression of TGF-β1 in suprabasal keratinocytes, appears to have dual effects. It suppresses the formation of benign skin tumors, but once tumors develop, it enhances their progression to a highly invasive spindle cell phenotype [2••]. These results suggest that the effects of TGF-β1 are biphasic: TGF-β1 acts early as a tumor suppressor, probably by inhibiting the proliferation of nontransformed cells, and it acts later as a tumor promoter by eliciting an epithelial to mesenchymal transition (EMT). Additional experiments have suggested that upregulation of TGF-β3 in the spindle carcinomas was responsible for maintenance of this invasive phenotype [2]. This is consistent with [*i*] TGF-β3 expression at sites in mouse embryos where epithelial-mesenchymal interactions are important, like the lung and palatal shelves [3,4]; and [*ii*] the abnormal lung development and cleft palate observed in TGF-β3 null mice [5]. Also consistent with an early tumor suppressive effect is the recent observation that TGF-β1-/- mice develop an accelerated progression of epithelial hyperplasia to colonic adenomas and cancers [6•]. The existence of dual effects for TGF-βs in tumor progression follows the observation that TGF-β-induced growth inhibitory responses and extracellular matrix responses may represent distinct processes in certain cell types. For example, overexpression of the antagonistic Smad, Smad7, in pancreatic carcinoma cell lines not only suppresses TGF-β1-mediated growth inhibition, but enhances the ability of TGF-β1 to induce matrix-associated transcriptional responses [7•].

The progression of epithelial tumors to an invasive metastatic state is often associated with EMT, downregulation of cellular adhesion molecules, elevated expression of metalloproteases, and increased motility and angiogenesis, all of which can be modulated by TGF-βs. It is therefore not surprising that the TGF-βs can also promote tumorigenesis by modulating these critical processes. In support of this view, elevated levels of TGF-βs are often observed in advanced carcinomas, and have been correlated with disease progression in several studies [8-13]. This suggests that secreting higher levels of TGF-β may provide an advantage to tumor cells. Both autocrine and paracrine signaling may be involved in conferring this selective advantage. While mutations in various components of the TGF-β signaling pathway have been observed in some carcinomas, particularly, colorectal cancers [14,15], an intact TGF-β signaling pathway is often retained in other malignancies as some tumors can exhibit increased invasiveness in response to exogenous TGF-β [16-21]. Moreover, in a recent study of a large cohort of human breast tumors, loss or low levels of the type II TGF-β receptor (TβRII) correlated with high tumor grade, but 60% of *in situ* and invasive breast carcinomas retained robust levels of TβRII expression by immunohistochemistry [22]. Finally, although Smad4 is frequently inactivated in pancreatic cancers [23,24], the Smad genes, which encode proteins that transduce TGF-β signals, are rarely mutated in most human carcinomas [25-30]. This suggests that after cells lose their sensitivity to TGF-β growth inhibition, autocrine TGF-β signaling may potentially promote tumor progression. In addition, TGF-βs produced in excess by tumor cells may act in a paracrine fashion on the peritumoral stroma, tumor neovessels, or the immune system, indirectly fostering tumor progression.

AUTOCRINE EFFECTS

Epithelial to Mesenchymal Transition (EMT)

Similar to keratinocytes [2••], TGF-β1 can also induce a rapid and reversible EMT in melanoma cells [31], and in both non-tumor [32] and Ha-Ras transformed [17•] mammary epithelial cells *in vitro*. In Ha-Ras mammary tumors, EMT appears to be initiated by TGF-β produced by peri-tumoral host cells and later maintained by autocrine TGF-β1 as the converted tumor cells themselves begin to secrete TGF-β1. The Ha-Ras tumor cells obtained after EMT *in vitro* or *in vivo* display loss of epithelial polarity, downregulation of E-cadherin, disruption of cell-cell adhesion, and invasive properties in several *in vitro* assays [17•]. Supporting the importance of autocrine TGF-β for the tumorigenesis of Ha-Ras mammary cells, introduction of dominant negative TβRII into these cells retarded tumor formation and prevented EMT *in vivo*. Moreover, introduction of the same construct into highly invasive murine colon carcinoma cells, reconstituted an epithelial phenotype *in vitro*, and inhibited both tumor outgrowth and the establishment of metastases [20•]. In colon cancer cells of low invasive potential and with naturally occurring mutations in the TβRII gene, re-expression of TβRII function restored tumor cell invasiveness [20•]. In another study, expression of a dominant negative TβRII in clones derived from a metastatic squamous carcinoma cell line prevented their spontaneous progression to a spindle phenotype *in vivo* [21]. Furthermore, approximately 90% of colon cancers with microsatellite instability (MSI) have inactivating mutations of TβRII [33] and MSI is significantly correlated with longer patient survival [34], suggesting that complete loss of TβRII in carcinomas may limit systemic metastases. Taken together, these results suggest that EMT, local tumor growth, and metastatic progression can be sustained by autocrine TGF-β signaling.

When tumors are grown in nude mice, TGF-βs made by host cells can induce responses in tumor cells with intact TGF-β signaling with the net effect of these tumor/host interactions being deleterious to the host. For example, MDA-231 human breast tumor cells secrete parathyroid hormone-related protein (PTHrP) in response to exogenous TGF-β1, metastasize to bone when injected into nude mice, and induce osteolysis and hypercalcemia resulting in host death. Transfection of these cells with dominant-negative TβRII, blocks TGF-β1-mediated stimulation of PTHrP production. Mice injected with these cells exhibited less osteolysis, higher body weight, lower serum calcium and PTHrP levels, and longer survival than mice injected with control MDA-231 cells [35•]. On the contrary, accelerated osteolysis and reduced host survival were observed when mice where injected with tumor cells transfected with a constitutively active TβRI, suggesting a possible role for TGF-β mediated responses in the pathogenesis of some adverse paraneoplastic syndromes.

Several recent studies have contributed to our understanding of the biochemical mechanisms by which transformed cells can lose autocrine growth inhibition but retain TGF-β-mediated responses that contribute to tumor progression. For example, oncogenic activation of the Ras pathway, acting via MAP kinases, causes phosphorylation of Smad2 and Smad3 at specific Erk consensus sites in the linker region between their DNA-binding and transcriptional activation domains. This results in loss of nuclear accumulation of Smad2/3 and silencing of TGF-β mediated antiproliferative responses [36••]. In nontransformed mammary cells, introduction of mutant Ras not only blocks growth inhibition by TGF-β, but also subverts this

pathway into one that can stimulate epithelial to mesenchymal transdifferentiation [17•,20•]. In MDCK epithelial cells, transfection of the missense mutations Smad2.D450E and Smad2.P445H, reported in primary colorectal and lung carcinomas, does not abolish TGF-β-mediated growth arrest. Instead, it increased both basal and TGF-β stimulated invasiveness, neither of which were prevented by overexpression of the inhibitory Smad7 [37•]. This suggests the existence of Smad 'gain-of-function' mutations that enhance malignant progression by mechanisms independent of TβRI and Smad phosphorylation. Another study has shown that Smad7 mRNA levels are increased in human pancreatic cancers compared to normal pancreas [7•]. Stable transfection of COLO-357 human pancreatic cancer cells with a Smad7 expression vector results in loss of TGF-β1-mediated growth inhibition and p21/Cip1 promoter activity. However, TGF-β1-induced plasminogen activator inhibitor-1 (PAI-1) promoter activity is maintained and, more importantly, basal PAI-1 promoter activity, PAI-1 mRNA levels, anchorage-independent colony growth, and tumorigenicity in nude mice, are all increased in the Smad7 transfected clones [7•]. This result suggests another potential mechanism, the overexpression of Smad7, for the segregation between antiproliferative and matrix-associated TGF-β responses. In addition, overexpression of Smad4 in colon carcinoma cells does not reconstitute TGF-β-mediated antiproliferative responses [38•,39], but inhibits cell adhesion and spreading, reduces the levels of urokinase plasminogen activator (uPA) and PAI-1, and prolongs tumor latency [39], suggesting an additional function for Smad4 in restraining genes involved in peri-tumor proteolysis and invasion. This is further supported by reports of homozygous deletion of TβRI or homozygous missense mutations of TβRII [40,41], each coexisting with deletions of Smad4 in individual tumors. The coexistence of these mutations in the same tumors would not be expected if the function of these two gene products (TβRII and Smad4 or TβRI and Smad4) was limited to a single common signal transduction pathway. Taken together, these studies suggest that [*i*] the threshold for loss of TGF-β antimitogenic effects is lower than that required to lose responses associated with cell adhesion, invasion, and metastases; [*ii*] not one but multiple biochemical mechanisms can contribute to the enhancement or unmasking of the tumor promoting effects of autocrine TGF-β; and [*iii*] some of these mechanisms may be independent of Smad function or TβRI phosphorylation. The identification of Smad-dependent and -independent genes causally involved in these TGF-β-mediated tumor promoting effects requires further research. Of note, Hocevar et al. [42•] recently reported c-Jun N-terminal kinase (JNK)-dependent TGF-β induced fibronectin expression in cell lines lacking the Smad4 gene or protein expression.

Increased Motility

TGF-β can stimulate the motility of many cell types *in vitro* [43-45], therefore suggesting that TGF-β production *in vivo* may enhance migration of tumor cells and metastatic potential. Indeed, cyclosporine treatment of lung adenocarcinoma cells results in increased cell motility and anchorage-independent growth *in vitro* as well as increased metastases *in vivo*, all of which can be blocked with neutralizing TGF-β1 antibodies [46]. These results suggest that *in vivo* tumor progression by cyclosporine is dependent on autocrine TGF-β1. In prostate cancer cells, TGF-β1 stimulates motility without affecting cell proliferation, suggesting that the effects on motility and proliferation may occur via different biochemical pathways [43].

Whether blockade of the Smad pathway, critical for TGF-β-mediated antimitogenic effects [47,48], is also critical for the effects of TGF-βs on cell motility is not clear. Some

evidence suggests that the latter may follow alternative signaling pathways perhaps in cooperation with activated oncogenes. Atfi et al. [49] recently reported that inactivating components of the JNK pathway, which via c-Jun regulates AP-1 activity, inhibits TGF-β mediated induction of 3TP-Lux, a reporter construct that contains Smad and AP-1 binding elements. Dominant-negative mutants of RhoA, Rac1, and Cdc42, GTPases that mediate cell shape, cytoskeletal organization, and motility, abolish TGF-β mediated transcription of AP-1 [49,50], suggesting that the Rho family of GTPases and the JNK pathway are essential components of TGF-β signaling responses. TGF-β1 can also upregulate integrin-linked kinase (ILK) [31], a protein associated with fibronectin production and increased cell motility. In another study, TGF-β1 treatment of NMuMG mouse mammary epithelial cells increased the expression of N-cadherin [51], which has been shown to increase motility of squamous cancer cells [52].

PARACRINE EFFECTS

Induction of Metalloproteases

Matrix metalloproteases (MMPs) play a critical role in the proteolytic degradation of basement membrane that is required for tumor invasion [53]. The expression of several MMPs, including MMP-2 [54] and MMP-9 [18,31,55], can be induced by TGF-β. Moreover, TGF-β1 has been shown to selectively induce MMP-9 activity in a subset of metastatic but not primary mouse prostate tumors, implying that this TGF-β1-induced response may be an important selection step in tumor progression [18]. There is also evidence that TGF-β increases MT-MMP-1 and MMP-9 expression in metastatic melanoma [31]. Although MMPs are listed separately, recent data implicate them strongly in the process of tumor-induced neovascularization [56], thereby suggesting that their upregulation might be an integral component of the TGF-β-mediated angiogenic processes discussed below.

Tumor Angiogenesis

It is generally accepted that solid tumors require an adequate blood supply in order to grow beyond a few millimeters in size. TGF-βs, particularly TGF-β1, have been shown to regulate new blood vessel formation both *in vitro* and *in vivo* by a combination of responses that include increased production of vascular endothelial growth factor (VEGF), facilitation of VEGF- and basic fibroblast growth factor (bFGF)-mediated capillary sprouting, inhibition of endothelial cell migration, and increased production of extracellular matrix, among others (reviewed in [57]). In most cells, TβRI/ALK-5 is the signaling receptor for TGF-β. However, in endothelial cells, it has been suggested that ALK-1 may also function as a type I receptor for TGF-β [58]. In addition to the type I, II, and III TGF-β receptors, endoglin is another integral membrane protein that binds TGF-β1 and TGF-β3, and is highly expressed in endothelial cells [59]. Although TGF-β effects appear to be mediated mostly by the receptor-specific Smad2 and Smad3 proteins [47,48], there is evidence that Smad5 is involved in TGF-β signaling in hematopoietic cells [60]. Targeted disruption of genes encoding various components of the TGF-β signaling pathway, including TGF-β1 itself [61], its receptors, TβRII [62], ALK-1 [63], endoglin [64], and one of its signal transducers, Smad5 [65], has each revealed that these proteins play an important role in vascular development. The phenotype of the TGF-β1 and TβRII knockout mice is virtually indistinguishable and is characterized by defective endothelial differentiation resulting in abnormal capillary tube formation [61,62]. In contrast, disruption of

ALK-1, endoglin, or Smad5 does not affect endothelial differentiation or vasculogenesis, but instead they all affect angiogenesis. In addition, endoglin-/- and Smad5-/- mice exhibit impaired vascular smooth muscle cell development. These results are consistent with previous reports demonstrating that TGF-β can regulate smooth muscle cell differentiation and migration *in vitro* [66•], thus contributing to pericyte recruitment and vessel stabilization. This hypothesis, as it applies to tumor angiogenesis, is somewhat challenged by the notion that the majority of intratumoral neovessels seem to lack periendothelial smooth muscle cells [67], suggesting that there may be additional roles for the TGF-βs in tumor angiogenesis. In that light, Higaki et al. [68] recently reported TGF-β1-mediated stimulation of phosphatidylinositol-3 kinase (PI-3K) activity and amino acid uptake in vascular smooth muscle cells, suggesting a direct anti-apoptotic role for TGF-β. Elucidation of the paracrine mechanisms driving TGF-β mediated tumor angiogenesis requires further investigation.

Further supporting TGF-βs' role in tumor angiogenesis, administration of a neutralizing TGF-β1 antibody to nude mice harboring CHO cell xenografts transfected with ectopic TGF-β1, inhibits both tumor growth and intratumor microvessel density [69]. In addition, a monoclonal antibody that blocks TGF-β1, -β2, and -β3 has been shown to suppress the growth of TGF-β1-overexpressing renal cancer xenografts [70]. In this study, the TGF-β blocking monoclonal abrogated factor VIII staining in the xenografts, suggesting an antitumor mechanism that targets endothelial cells [70]. Furthermore, TGF-β1 and PAI-1 have been shown to inhibit the conversion of plasminogen to the antiangiogenic molecule angiostatin in medium conditioned by human pancreatic cancer cells [71]. This suggests an additional proangiogenic mechanism for TGF-β by interferring with the production of endogenous inhibitors of endothelial cell proliferation. Finally, high levels of TGF-β1 mRNA correlate strongly with high microvessel density in breast tumors, and each of these factors is associated with poor patient outcome [72].

Host Immunosuppression

TGF-β1 and TGF-β2 are potent immunosuppressants [73]. Thus, elevated levels of TGF-βs secreted by tumors could potentially inhibit immune effector cells and favor tumor progression. In support of this idea, Torre Amione et al. [74] demonstrated that, unlike parental tumor cells, fibrosarcoma cells transfected to express 10 ng/ml TGF-β1 *in vitro* are unable to induce cytotoxic T lymphocyte (CTL) responses and can escape immune recognition. Likewise, EMT6 mammary tumor cells, which produce high levels of TGF-β1, can inhibit CTLs *in vivo*. Transfection of these cells with IL-2, a known T-cell growth factor, can reverse this TGF-β1 effect and induce tumor rejection [75]. This result suggests that, by dampening the generation of tumor reactive T cells, TGF-β can promote tumor viability. There is also evidence that overexpression of the soluble TβRII extracellular domain in thymoma cells can prevent the progression of unmodified thymoma cells when injected near the primary tumor inoculation site [76], further suggesting that secretion of soluble TβRII by these cells is sufficient to restore tumor specific cellular immunity and mediate partial tumor rejection. Overall these results are consistent with the phenotype of TGF-β1 null mice which die shortly after birth as a result of widespread inflammation and multiorgan T cell infiltration and necrosis [77].

In addition to inhibiting CTL responses, TGF-βs can modulate other immune functions that may favor tumor progression. For example, CHO cells transfected with an expression vector

encoding latent TGF-β1, when injected into nude mice, can decrease mouse spleen natural killer activity and rapidly form tumors [78]. Antagonizing TGF-βs by intraperitoneal injection of an antibody that neutralizes TGF-β1, -β2, and -β3 has the opposite effect. It prevents tumor and metastases formation by MDA-231 human breast carcinoma cells, and markedly increases natural killer activity of mouse splenocytes [79]. Consistent with this TGF-β-mediated immunosuppressive effect, reduced immune function has been observed in animals bearing TGF-β overexpressing tumors [80] as well as in patients with glioblastoma, a common type of brain tumor that frequently overexpresses TGF-β2 [81].

The studies mentioned above suggest that tumor cell secreted TGF-βs may block the efferent function of immune effectors at sites of tumor implantation. Other reports, however, suggest tumor cell TGF-βs may modify the afferent component of the immune response and confer antitumor immunity. Stable infection of breast and glioma tumor cells with antisense TGF-β1 and antisense TGF-β2 retroviruses, respectively, has been shown to restore the immunogenicity of these tumor cells when injected into immunocompetent animals. Furthermore, they induce a partial rejection of unmodified less immunogenic established wild-type tumor cells [82,83]. In both of these studies, *in vitro* and *in vivo* CTL activity was markedly increased in medium conditioned by antisense TGF-β-infected cells and/or in mice injected with tumor cells bearing the antisense compared to tumor cells infected with a control vector. These studies have therapeutic implications for the use of an antisense TGF-β based approach as a means of adoptive immunotherapy against TGF-β overproducing tumors.

Alternative Views and Conclusions

A tumor-permissive role for the TGF-βs may not apply to all solid tumors. Indeed, transfection of an antisense TGF-β1 expression vector into FET and CBS well-differentiated human colon cancer cells has been shown to enhance tumor formation in nude mice [84,85], supporting the notion that, in some fully transformed cells, endogenous TGF-β1 can continue to mediate a tumor suppressor function. In a recent report, mice bearing transplanted gallbladder Mz-Cha-2 tumors showed inhibition of angiogenesis and leukocyte-endothelial cell interactions at a distant cranial site and 3-fold higher levels of circulating TGF-β1 compared to tumor-free mice [86]. This reduction in microvessel density and leukocyte rolling were reversed by systemic administration of a TGF-β1 neutralizing antibody, suggesting a negative role for TGF-β1 in early neovascularization. Moreover, in a recent survey of 104 *in situ* and invasive primary breast carcinomas, 40/45 (89%) tumors with low invasive potential and low proliferation rate exhibited high levels of TβRII by immunohistochemistry [22]. Whether autocrine TGF-β signaling is causally associated with the observed low proliferation and invasiveness in this subset of breast tumors is a question that remains unclear.

Nonetheless, the potential tumor promoting effects of TGF-β provide novel molecular targets for interventions aimed at altering the natural history of solid tumors. The lack of an obvious physiological role for TGF-β signaling in post-developmental normal physiological states suggests that these interventions may in fact be tumor-specific and spare the tumor host from undue toxicity. Several approaches have been proposed and include the use of [*i*] blocking antibodies against TGF-β1, TGF-β2, and TGF-β3; [*ii*] soluble ectodomains of the type II and III

TGF-β receptors, which would sequester TGF-β isoforms at tumor sites and prevent binding to cognate receptors [87,88]; and [*iii*] adenovirus encoding inhibitors of TGF-β signaling [89], to name a few. The theoretical and logistical strengths and limitations of these approaches are beyond the scope of this review. Nonetheless, these represent tools that, if effective in blocking TGF-β action, will allow us to address the net effect of autocrine/paracrine TGF-β signaling at early and late stages of transformation and cancer progression.

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